IMMUNOLOGICAL RESPONSE OF RATS TO *MYCOPLASMA ARTHRITIDIS*

by

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ABSTRACT

IMMUNOLOGICAL RESPONSE OF RATS TO MYCOPLASMA ARTHRITIDIS

A sensitive assay for titration of antibodies which inhibit the metabolism of arginine by M. arthritidis was developed. Sera collected from rabbits immunized with M. arthritidis inhibited arginine metabolism when diluted 1:1600. The addition of fresh guinea pig serum enhanced the metabolic inhibition (MI) titer at least sixteen fold while heated guinea pig serum did not affect the titer of the rabbit antiserum. It was shown that rats did not produce detectable metabolic inhibiting antibodies to M. arthritidis, but did produce complement fixing antibodies in high titers. Preliminary experiments disclosed that uninoculated rats could be passively protected against an intravenous challenge of live M. arthritidis when given serum from rats that had recovered from polyarthritis. The rat serum used had a high titer of complement fixing antibodies and no detectable MI antibodies.

Rats were injected intravenously with varying numbers of M. arthritidis. Rats inoculated with the larger doses of organisms developed polyarthritis to a greater extent than those injected with smaller doses. It was shown that complement fixing antibody occurred in the sera of rats injected with M. arthritidis within three days after inoculation. Sera from rats which developed polyarthritis

generally fixed complement at a greater dilution than sera of rats which did not develop polyarthritis.

It was also shown that rats which had recovered from polyarthritis were resistant to intravenous challenge with M. arthritidis. Only one of fourteen rats inoculated intravenously with M. arthritidis developed polyarthritis when challenged.

INTRODUCTION

Recently it was conclusively shown that mycoplasma cause disease in man. In 1962, the agent of cold-agglutinin-positive atypical pneumonia was shown to be a species of mycoplasma (Chanock et al., 1962).

Mycoplasma in man have been associated with nongonococcal urethritis (Ford et al., 1962, 1963; Freundt, 1953; Klieneberger-Nobel, 1960; Melen and Linnros, 1952; Salaman et al., 1946; and Shepard, 1954), leukemia (Barile et al., 1966; Girardi et al., 1965; Hayflick and Koprowski, 1965; and Hummeler et al., 1965), erythema multiforme, conjunctivitis and urethritis (Stevens-Johnson syndrome, Katz et al., 1967 and Sieber et al., 1967), nonspecific urethritis, arthritis and conjunctivitis (Reiter's syndrome, Nicol and Edward, 1953; Oates et al., 1959; Schaffarzick and Mankle, 1953; and Wallerstein et al., 1946), exudative pharyngitis (Mufson et al., 1965), tonsillitis (Mufson et al., 1965), and arthritis (Ford, 1960; Jonsson, 1961; Wallerstein et al., 1946). Conclusive proof as to their direct involvement in these diseases is lacking.

Polyarthritis is a common manifestation of a mycoplasmal infection in lower animals. It occurs in the cow (Moulton et al., 1956), goat (Cordy and Adler, 1960), sheep (Cordy and Adler, 1960), fowl (Adler, 1959; Olson, 1959), pig (Aho et al., 1966; Lecce, 1960),

mouse (Klieneberger, 1940; Sabin, 1939), and rat (Collier, 1939b; Preston, 1942; and Woglom and Warren, 1938a). Polyarthritis often occurs in Reiter's syndrome and Stevens-Johnson syndrome in man, but absolute proof of the causative agent of this disease in man is lacking.

Since polyarthritis is such a common manifestation of mycoplasma infection and since this disease occurs naturally in rats, a study of this manifestation could contribute much to our understanding of the pathogenesis of mycoplasma disease in animals and possibly in man. Polyarthritis can be produced in rats following intravenous injection of a large dose of Mycoplasma arthritidis. Thus we have a convenient experimental model for the study of mycoplasma infection. The rat is a useful experimental animal in which to study the parameters of mycoplasma infection.

It was decided that an investigation of polyarthritis caused by M. arthritidis in rats should be carried out. Serological responses of the rats to infection by M. arthritidis were given special emphasis in the study reported in this thesis.

LITERATURE REVIEW

I. POLYARTHRITIS IN RATS

In 1938 Woglom and Warren described an agent which they thought to be a virus. The agent was encountered in a transplantable rat sarcoma and caused extensive necrosis when injected subcutaneously into experimental laboratory rats. These workers were unable to grow the agent in vitro, but were able to transfer the agent to other rats by injection of ten per cent extracts of the abscess in physiological saline. When the extract was injected intravenously into rats, their feet became edematous and reddened within three days and abscesses developed several days later. The agent passed through a Berkefeld N filter, a filter known to retain most bacteria.

Klieneberger (1938) devised a medium which she used to isolate mycoplasma (Nowak, 1929) from the enlarged submaxillary gland of a rat. She designated the organism L4 and showed the organism to be different from other mycoplasma by agglutination studies. Using her special medium, Klieneberger was able to isolate an organism similar to L4 from the lesions which developed in rats after injection of Woglom's pyogenic agent (Klieneberger, 1938, 1939). Woglom and Warren later confirmed the presence of mycoplasma in the lesions caused by their pyogenic agent (Woglom and Warren, 1939).

In a series of papers, Collier and others working with him (Beeuwkes, 1940; Beeuwkes and Collier, 1942; Collier, 1939a; 1939b; 1939c; 1939d; 1940; and Collier and Esseveld, 1938) reported a spontaneous polyarthritis of wild rats which could be transmitted to experimental laboratory rats. The agent described was identical in morphology to the L4 organism described by Klieneberger (1940) and exhibited a similar pathogenicity.

Findlay et al. (1939) also isolated a mycoplasma from the affected joints of experimental laboratory rats which had contracted polyarthritis naturally. They identified the organism as "L7," but the organism was subsequently found by Klieneberger (1940) to be serologically identical with L4.

The pathological changes of polyarthritis in rats were described by Findlay et al. (1939). In the early stages there was infiltration around the joint with polymorphonuclear leucocytes and large mononuclear cells. Later, fibroblasts appeared and finally the joint cavity was invaded with disappearance of articular cartilage and absorption and disorganization of the epiphysis.

A disease in members of a large breeding stock of rats used for experimental purposes was described by Rhodes and van Rooyen (1939). The symptoms of the disease were similar to those caused by Klieneberger's L4. Rhodes and van Rooyen did not attempt to cultivate the organism on a medium which would permit growth of mycoplasma, but were able to transmit the disease by injecting pus

from infected rats into the foot pads of healthy rats. An attempt was made to transmit the infection in the laboratory by placing infected rats in the same cage as healthy ones, but the infection did not spread.

In 1941, Sabin suggested the etiological agent of spontaneous polyarthritis in the rat (L4 of Klieneberger, 1940 and L7 of Findlay et al., 1939) be called Murimyces arthritidis. This name was not as well accepted as L4.

The genus Mycoplasma was established by Edward and Freundt (1965) to include the pleuropneumonia-like organisms originally described by Nocard et al. (1898). Edward and Freundt proposed the name Mycoplasma arthritidis for the agent of polyarthritis in rats.

Preston (1942) noted that arthritis was a frequent complication of animal diseases known to be caused by mycoplasma but was not able to culture mycoplasma from joint fluid of humans with rheumatoid arthritis. In studies using M. arthritidis, he described pathological changes in a rat. Following intravenous injection of M. arthritidis, the joints become swollen and edematous; the area within and around the joint capsule becomes purulent; and the synovial membrane, cartilage and bone around the joint often become involved in an acute suppurative process. In this respect, the lesion more closely resembles that seen in pyogenic joint infections of man rather than that of rheumatoid arthritis. The abscess around the joint may rupture and heal, leaving a relatively good function

in the joint. Ankylosis may occur in joints which have undergone marked destruction of the cartilage.

Parks and Wrigley (1951) also used histiological techniques to show that rheumatic diseases of man were different from experimental polyarthritis in rats.

The role of mycoplasma in genitourinary and joint diseases was discussed by Dienes et al. (1948). The presence of fever, chills and malaise, along with infection of the joints of several male patients from which mycoplasma had been isolated from the genitourinary tract led to the suggestion that mycoplasma produce a generalized human infection with joint localization.

II. IMMUNE RESPONSE OF RATS

Although the histiological aspects of rat infections of M. arthritidis have been discussed thoroughly in the literature, little has been reported on antibody response and immunity of the rat to challenge with M. arthritidis.

Resistance to challenge with M. arthritidis was well established by the seventh day after subcutaneous or intravenous inoculation of M. arthritidis (Woglom and Warren, 1938a). Sera collected from rats inoculated one to two weeks previously with the agent which caused polyarthritis would not neutralize the agent when combined with varying numbers of the organism and incubated at 37°C for ninety minutes. Rats inoculated intratesticularly with the immune

serum-mycoplasma mixture reacted similarly to normal rats injected with mycoplasma which had not been mixed with rat antiserum.

In 1939, Collier (1939b) reported that both laboratory and field rats immunized with the agent of polyarthritis were immune to challenge with the same organism. Even rats which did not have the symptoms of polyarthritis were immune to challenge with the organism.

As with Woglom and Warren (1938a), Collier could not find neutralizing antibodies in the serum of immune rats.

Two rat polyarthritis strains of M. arthritidis were recently investigated by Klieneberger-Nobel (1960). An encapsulated abscess was formed at the site of subcutaneous inoculation of the organism. The abscess increased in size during the first three to four weeks and regressed slowly. Complement fixation antibody titers rose to 1:2500 during the first four to five weeks after inoculation and then declined gradually during the next eighteen weeks.

Lemcke (1961) used a strain of mycoplasma which was less virulent than the organisms tested by Klieneberger-Nobel (1960). The complement fixation titer reached a maximum of 1:320 four to five weeks after subcutaneous injection of the organisms. She attempted to isolate mycoplasma from rats which had been inoculated with the organisms. There was a correlation between the presence of mycoplasma demonstrated by culture and the occurrence in the blood of antibodies specific for the infecting strain. When mycoplasma were

present in the rat, complement fixing antibodies specific for that organism could be demonstrated. The titer of the serum was directly related to the severity of the infection. The titer increased as the severity of the disease increased.

Lemcke (1961) suggested that since there was a direct relationship between the presence of mycoplasma in the rat and antibody specific for that organism, then specific antibody found in man could be a significant indicator of mycoplasma infection.

It is evident from this review of literature that more information on the role and significance of antibody in rat polyarthritis caused by mycoplasma is needed to more fully understand polyarthritis in rats.

MATERIALS AND METHODS

I. MEDIA

A. Media for Maintenance of Mycoplasma

The medium used for isolation and maintenance of mycoplasma organisms was described by Taylor-Robinson et al. (1966).

Another solid medium, base agar, was prepared by a modification of the method described by Cole and Pease (1967). It consisted of 1% proteose peptone No. 3 (Difco), 0.125% yeast extract (BBL), 1% Lab-Lemco (Oxoid beef extract), 0.5% sodium chloride and 1% Ionagar No. 2 (Oxoid). The mixture was adjusted to pH 7.8 with 40% sodium hydroxide and autoclaved at 121°C for 15 minutes, after which it was placed in a 56°C water bath for two hours to allow any precipitate which developed to settle. The supernatant was decanted into a sterile flask and 15% unheated horse serum, 2.5% fresh yeast extract and 1000 units/ml Penicillin G were added. The medium was mixed thoroughly and poured into sterile plastic petri dishes. The plates were stored in metal containers at 4°C until used. Fresh yeast extract was prepared by a modification of the method described by Taylor-Robinson et al. (1963). Baker's yeast (100 grams) was added to 300 ml of distilled water. The mixture was boiled until one-third of the original volume remained. After centrifugation at 25,000 x g for 15 seconds, the supernatant yeast extract was sterilized by

filtration through a Seitz filter. The yeast extract was stored at -20°C until used.

B. Medium for Metabolic Inhibition (MI) Tests

The medium used for the metabolic inhibition test was a modification of that described by Taylor-Robinson et al. (1966). It consisted of 7 parts of Difco PPLO broth, 2 parts of unheated horse serum, 1 part of fresh yeast extract, 1% L-arginine HCl, 0.002% phenol red and 1000 units/ml Penicillin G. The arginine broth medium was adjusted to pH 7.2 with hydrochloric acid.

C. Rabbit Infusion Broth

The organisms used for immunization of rabbits were grown in a rabbit muscle infusion broth. The infusions were prepared by the method described by Taylor-Robinson et al. (1963). Eight ml of distilled water were added to each gram of rabbit muscle. The mixture was finely ground in a Waring blender. After blending, the mixture was heated to boiling and the infusion was cooled and filtered through gauze and then filter paper. Peptone (1%; Difco) and 0.5% sodium chloride were added and the pH adjusted to 7.8 with 40% sodium hydroxide or 10% hydrochloric acid. The preparation was sterilized by autoclaving at 121°C for 15 minutes. Two ml of cholesterol (Fisher Scientific Company) solution were added to each 100 ml of rabbit infusion. The steroid was prepared by dissolving 100 mg of cholesterol in a small quantity of absolute ethanol and adding

the solution to 100 ml of hot distilled water. Rabbit infusion was enriched with 7.5% normal rabbit serum.

D. Media for Biochemical Identification of Mycoplasma

Unknown mycoplasma were tentatively identified by their biochemical properties.

"Incomplete medium" consisted of 80 to 85% Difco PPLO broth, 10% unheated horse serum, 5% fresh yeast extract and 1000 units/ml of Penicillin G. For solid media PPLO broth was replaced with Difco PPLO agar.

E. Glucose Broth

Glucose broth was prepared by adding 10 ml of Seitz filtered 10% glucose and 2 ml of 1:1000 phenol red to 90 ml of sterile incomplete medium. The broth was distributed to sterile screw-cap culture tubes (15 x 100 mm) in 2 ml amounts. Three drops of a four-day culture of mycoplasma in PPLO broth were used to inoculate the glucose broth. The tubes were incubated aerobically 4 to 7 days and checked for color change of the indicator. The test was considered positive if the indicator changed in color from red to yellow.

F. Arginine Broth

Arginine broth was prepared by mixing 10 ml of sterile (121°C for 15 minutes) 10% L-arginine HCl with 90 ml incomplete medium. The arginine broth was dispensed into sterile screw-cap culture tubes in 2 ml amounts and inoculated as for the glucose broth. After

4 to 7 days of incubation under aerobic conditions, a drop of the arginine broth culture was mixed with a drop of Nessler's reagent on a petri dish. An orange-brown coloration indicated arginine had been deaminated and ammonia had been released into the medium.

G. Egg Yolk Agar

Egg yolk agar (McClung and Toabe, 1947) was prepared by mixing 10 ml of Oxoid egg yolk with 90 ml of incomplete medium. The molten agar was poured into plastic petri dishes and placed in a 37°C incubator with the lids raised to allow evaporation of excess moisture. The agar was inoculated with two drops of four-day-old mycoplasma broth culture or with organisms growing on a small section of base agar. The plates were incubated at 37°C for 4 to 7 days under aerobic conditions and checked for lipolytic activity indicated by an oily film on the agar surface.

H. Benzidine Blood Agar

Benzidine blood agar was prepared by adding 4 ml sterile sheep blood and 1 ml of 1% benzidine in acetic acid to 95 ml of incomplete agar medium. The benzidine blood agar plates were poured and inoculated as for the egg yolk agar plates. After 4 to 7 days of aerobic incubation at 37°C the plates were checked for growth of the mycoplasma and production of peroxide as indicated by a brown-black color beneath or around the mycoplasmal growth.

I. Triphenyl Tetrazolium Chloride Agar

Triphenyl tetrazolium chloride (TTC) agar (Somerson and Morton, 1953) was prepared by mixing 1 ml of 0.5% TTC with 99 ml of incomplete medium. Agar plates were prepared and inoculated as described previously. Each organism to be tested was inoculated onto two TTC agar plates. One was incubated aerobically at 37°C and the other anaerobically at the same temperature. The TTC plates were checked for growth of the mycoplasma and for reduction of TTC in or around the mycoplasma colonies. Triphenyl tetrazolium chloride which is colorless in the oxidized state becomes red when reduced.

J. Mycoplasma Strains Used

Several strains of mycoplasma isolated from the urogenital tract of man cause polyarthritis when injected intravenously into rats. These organisms have been referred to in the literature as M. hominis, type II.

Lemcke (1964), Lemcke and Csonka (1962) and Pease (1965) used complement fixation and gel double diffusion tests respectively to demonstrate antigenic identity of M. arthritidis and M. hominis, type II. Edward (1954) and Edward and Freundt (1956) found that these organisms were the same on the basis of physiology and morphology. Cole et al. (1967) reported that the virulence of M. hominis, type II and M. arthritidis strains maintained on artificial media was identical. M. hominis, type II and M. arthritidis are also

indistinguishable by the agar column hybridization technique used to estimate genetic relatedness (McGee et al., 1967). Recently Edward and Freundt (1965) proposed that M. hominis, type II be reclassified as M. arthritidis.

Since M. arthritidis and M. hominis, type II are indistinguishable on the basis of antigenicity, physiology, morphology, virulence and genetic homology, the two species will be considered the same by the author. M. hominis, type II will be referred to as M. arthritidis in this thesis.

M. arthritidis, strain 14152, and M. arthritidis, strain 14124, were obtained from the American Type Culture Collection (Rockville, Md.). Frozen cultures of the organisms were used as stock cultures in these studies.

M. arthritidis, strain DL, was isolated by Laura Rowland and James Cahill (University of Utah, Salt Lake City, Utah) from the joint of a Long-Evans, strain L, black and white rat suffering from polyarthritis.

M. arthritidis, strain 158, was obtained from Dr. Michael F. Barile (Division of Biologic Standards, Bethesda, Md.) and was passaged ten times by subcutaneous inoculation in white laboratory rats.

K. Rabbit Hyperimmune Sera

The techniques of Pease and Laughton (1965) and Morton and Roberts (1966) for production of rabbit antisera were modified. One

or two liters of rabbit infusion broth were inoculated with 100 to 200 ml of a 3 to 4 day culture of mycoplasma. The culture was incubated at 37°C for three days or until growth was noticeable. The organisms were concentrated in a Servall centrifuge at 27000 g and washed three times with saline (0.85% sodium chloride). The organisms were resuspended in 6 ml of saline and subjected to ultrasonic vibration using a sonifier (Branson Sonifier, model S-75) set on the one or two position and run at 2.5 to 3 milliamperes for 2 to 3 minutes. Hyperimmune rabbit sera was prepared either by the Pease (1965) or Morton and Roberts (1966) methods of immunization.

The Pease method consisted of two subcutaneous injections (each 0.5 ml of antigen + 0.5 ml of Freund's complete adjuvant) followed in two weeks by a graded series of intravenous injections of antigen alone (0.5, 0.75 and 1 ml) over a three-day period. The animals were test bled ten days following the final intravenous injection and then injected intramuscularly with 0.5 ml of antigen suspended in 0.5 ml of Freund's complete adjuvant when necessary.

The Morton method employed injections of 0.5 ml of antigen suspended in 0.5 ml of Freund's complete adjuvant into the hind footpads, followed in three weeks by injections (0.5 ml of antigen + 0.5 ml of complete Freund's adjuvant) into muscles of the shoulder region. Two weeks later the animals were test bled and when necessary, the rabbits were injected subcutaneously with 0.5 ml of antigen suspended in 0.5 ml of Freund's complete adjuvant.

L. Guinea Pig Serum

One ml aliquots of guinea pig serum (BBL) were dispersed into small serum bottles and stored at -20°C. Fresh guinea pig serum was used as a source of a heat labile accessory factor in the metabolic inhibition test and as a source of complement in the complement fixation test.

M. Absorbed Rabbit Immune Serum

Absorbed rabbit serum was prepared by mixing equal volumes of M. arthritidis, strain 14152 antigen and rabbit hyperimmune antiserum. The antigen was adjusted such that a 1:20 dilution gave an optical density difference of 0.32 on a Bausch and Lomb Spectronic 20 at wavelength 540 millimicrons. The spectrophotometer was set to zero with a water blank. The suspension was stored 4 to 5 days at 4°C, after which the particulate material was removed by centrifugation. The supernatant fluid was diluted 2:3, giving a final dilution of absorbed antiserum of 1:3. The absorbed rabbit serum was then heated at 56°C for 15 minutes.

N. Indirect Hemagglutination Test

An indirect hemagglutination test for the quantitation of antibody to mycoplasma has been developed by Taylor-Robinson et al. (1965) based on the procedure described by Friedman and Bennett (1957) for the detection of adenovirus antibodies. The procedure involved tanning of sheep erythrocytes, sensitizing the cells with

mycoplasma antigen and freezing the cells in a dextrose-lactose solution.

The test is carried out in the following manner: Mycoplasma used as antigen were grown in PPLO broth. After 3 to 4 days of incubation at 37°C the suspension of organisms was concentrated in a Servall centrifuge at 27,000 x g and the antigen resuspended in buffered saline (pH 7.2) containing 0.2% formalin. The organisms were subjected to ultrasonic vibration using a sonifier (described previously). Fresh sheep erythrocytes were washed three times in buffered saline and "tanned" by incubating a 4% suspension with an equal quantity of 1:20,000 tannic acid (Fisher Scientific Company) in buffered saline for ten minutes at 37°C. After washing the tanned cells three times in buffered saline, a 2% suspension was prepared in buffered saline. The antigen, diluted 1:40 gave an optical density difference of 0.32 on a Bausch and Lomb Spectronic 20 at wavelength 540 millimicrons. The spectrophotometer was set to zero with a water blank. The antigen was diluted in a two-fold series (starting with 1:8) in buffered saline. Aliquots of the dilutions were mixed with equal volumes of the 2% washed tanned cell suspension. After 20 minutes of incubation in a water bath at 37°C the suspension was centrifuged at 1500 rpm for 10 minutes. The cells were resuspended in buffered saline with 1:200 normal rabbit serum, inactivated at 56°C for 20 minutes. The suspension was again centrifuged at 1500 rpm for ten minutes and resuspended in the 1:200

normal rabbit serum to produce a 2% suspension of sensitized tanned sheep erythrocytes. For controls, tanned sheep erythrocytes were treated by the same procedure except that buffered saline was substituted for antigen.

Rabbit antiserum was heated at 56°C for 30 minutes and diluted in a two-fold series (starting with 1:50) in 1:200 normal rabbit serum. Sensitized sheep erythrocytes (0.5 ml) were added to 0.5 ml of serum; incubated 2 hours at 37°C; and then overnight at 4°C. The serum titer was considered to be the highest dilution of antiserum which caused complete agglutination of the sensitized sheep erythrocytes.

In order to obtain reproducible results, and to reduce the time required to set up the tests, a large quantity of sensitized sheep erythrocytes was preserved by a modification of the method described by Hubert et al. (1963).

A 4% suspension of sensitized tanned erythrocytes was prepared in 1:200 normal rabbit serum. The suspension was mixed with an equal volume of dextrose-lactose solution; the final concentration of the dextrose and lactose in the solution being 5% and 7.5% respectively. Aliquots of the sugar-erythrocyte mixture were placed in glass tubes and shell frozen in acetone. The cell suspensions were stored at -70°C until needed, at which time they were thawed rapidly by agitation in a 37°C water bath and centrifuged at 1500 rpm for 10 minutes. The erythrocytes were washed once in 1:200 normal rabbit

serum in buffered saline and resuspended to yield a 2% concentration of sensitized sheep erythrocytes in 1:200 normal rabbit serum. The indirect hemagglutination test was set up as described in the titration of the antigen.

O. Metabolic Inhibition (MI) Test

The method is a modification of the metabolic inhibition test described by Purcell et al. (1966). All tests were performed in two ml screw-cap serum vials. Rabbit antiserum was diluted 1:10 or 1:50 in PPLO broth and then heated at 56°C for 15 minutes.

The test was performed as follows: 0.25 ml of broth medium (with arginine) was placed into each tube. Serum samples (0.25 ml) were added to the first tube of each series and serial two-fold dilutions were carried out. A broth suspension of organisms (1,000 colony forming units in 0.2 ml) was then added to all tubes except medium control tubes. When guinea pig serum was used as a source of heat labile accessory factor, it was delivered in 0.1 ml amounts to each tube. The total volume was brought to 0.9 ml by the addition of 0.35 ml (0.25 ml when guinea pig serum was used) of broth medium. The medium control tubes received an additional 0.2 ml to bring the volume of fluid in all tubes to 0.9 ml. The tubes were incubated without agitation at 37°C under aerobic conditions. Serum titers were recorded when the tubes which contained organisms but no antiserum had changed from orange to red (approximately 72 hours). The highest serum dilution which prevented a color change

was recorded as the end point. The addition of specific antiserum to the medium inhibits mycoplasma growth and subsequent metabolism of arginine. This is manifested by failure of the indicator to change color.

P. Complement Fixation Procedure

Complement fixation titers of the sera were determined by the one-fifth quantitative serum test of Kolmer and Boerner (1945).

Antigen was prepared as described in the indirect hemagglutination procedure. The antigen was diluted to give an optical density difference reading of 0.16 on a Beckman model DB at wavelength 540 millimicrons. The spectrophotometer was set to zero with a water blank. All dilutions in the test were made with modified barbital buffer, as described by Campbell et al. (1962).

Q. Quantitative Complement Fixation Test

Serial two-fold dilutions of the rat serum (1:10 to 1:40,960) contained in 0.2 ml were prepared in MBB. Antigen (0.1 ml) was added to each tube. Ten to thirty minutes later, two units of complement contained in 0.2 ml were added. The tubes were incubated overnight at 4°C, followed by 10 to 15 minutes in a 37°C water bath. Two units of hemolysin (0.1 ml) and 0.1 ml of a 2% sheep erythrocyte suspension was added to each tube. The tubes were shaken and incubated in a 37°C water bath until the erythrocytes in the control tube containing complement hemolysin and erythrocytes were

completely lysed. The reciprocal of the greatest dilution was considered the titer of the serum.

R. Preparation of Frozen Cultures for Metabolic Inhibition Tests

All strains of mycoplasma were grown on PPLO or base agar. Isolated colonies were picked and replated two or three times to insure a pure culture of mycoplasma. A section of base or PPLO agar (approximately one inch square) with young colonies of the organism was placed in arginine broth and incubated for 1 to 2 days at 37°C under aerobic conditions. One ml aliquots of the growing culture were placed into 2.5 ml serum vials and stored at -20°C until used.

S. Determination of the Colony Forming Units of Mycoplasma

The number of colony forming units (CFU) was determined by a modification of the method described by Miles and Misra (1938). The procedure consisted of counting colonies which developed from measured drops of culture dilutions seeded on well-dried agar plates. Serial ten-fold dilutions were made of the suspension (10^{-1} to 10^{-5}). PPLO and base agar plates were placed in an incubator for 15 to 30 minutes with the lid raised to dry the agar enough to absorb a 0.005 ml drop of suspension in 15 to 20 minutes. A Yale disposable 25g 5/8" needle on a glass 1 ml syringe delivers approximately 200 drops/ml, or 0.005 ml/drop. A series of three drops for each dilution was placed on the agar plate. The plates

were incubated at 37°C for 3 to 4 days or until colonies had formed. The number of viable organisms was determined by taking the average of the three counts of that dilution which had 30 to 50 colonies per drop, multiplying by the number of drops in 1 ml, then by the dilution factor.

T. Isolation of Mycoplasma From Rats

A colony of Long-Evans, strain L, black and white rats which frequently has outbreaks of polyarthritis was tested for the presence of mycoplasma organisms. Blood was collected by cardiac puncture from thirty-three adult male rats weighing approximately 500 grams. The serum was harvested after 24 hours at 4°C and stored at -20°C for future determination of antibody titers. Five of the rats were exsanguinated and autopsied. Samples of lung, trachea, bladder, cecal material, liver, spleen, joint, axillary or inguinal lymph nodule, heart and kidney were minced and streaked onto base agar and placed into PPLO broth. Throat and nasal mucosa swabs were also streaked onto base agar and placed into broth. All cultures were incubated aerobically at 37°C for 4 to 5 days and observed for growth of mycoplasma. Representatives of the various types of mycoplasmas isolated from each culture were streaked onto base agar. If more than one colonial type were present, representative colonies of each type were again streaked onto base agar. These cultures on base agar were used to inoculate PPLO broth which was used as the inoculum for biochemical tests for identification of the organisms.

U. Intravenous Inoculation of Rats with Mycoplasma arthritidis

A broth culture of M. arthritidis, strain 158, was concentrated by centrifugation in a Servall centrifuge at 27,000 x g and resuspended in 8.5 ml of PPLO broth. The suspension was divided into two tubes and was stored at -20°C. The number of CFU in one tube, containing 0.5 ml, was determined using the method of Miles and Misra (1938). The other suspension was thawed immediately prior to inoculation and dilutions were prepared such that 0.5 ml contained approximately $2x10^9$, $2x10^8$, $2x10^7$, or $2x10^6$ CFU. The number of CFU of these dilutions was found to be $6x10^9$, $6x10^8$, $6x10^7$, and $6x10^6$ for 0.5 ml when counted immediately prior to injection into rats.

Holtzman white male rats weighing 100 to 120 grams were divided into five groups of seventeen animals and placed in metal cages with five or six animals per cage.

The mycoplasma suspensions ($6x10^9$, $6x10^8$, $6x10^7$, or $6x10^6$ CFU in 0.5 ml) or 0.5 ml of PPLO broth were injected into rats via the caudal vein.

Polyarthritis was determined by estimating the swelling of each joint and the paralysis of each limb. The swelling was recorded on a scale of 1 to 4 with minimal swelling of the joint, 1, and maximum swelling with possible accumulation of pus, 4. Paralysis and partial paralysis were 1 and 0.5 point respectively for each limb involved. The scores for each rat were totaled and recorded as the degree of polyarthritis. The severity of polyarthritis was recorded 3, 4, 5,

8, 10 and 14 days following injection of the mycoplasma, and once every week, thereafter, for eight weeks.

Blood samples were collected from each rat (via cardiac puncture) two days prior to inoculation of the animals, and 4, 8, 14 and 21 days following inoculation. The animals were bled from the orbital sinus once a week thereafter for approximately four months. The sera were harvested after overnight storage at 4°C and diluted 1:5 with modified barbital buffer. The samples were finally stored at -20°C until used in metabolic inhibition, indirect hemagglutination or complement fixation tests.

RESULTS

I. METABOLIC INHIBITION

Taylor-Robinson et al. (1966) have shown that neither heated nor unheated guinea pig serum have any effect on the metabolic inhibition titer of antisera to M. pneumoniae, strain FH, and M. fermentans; whereas, the inhibitory titer of rabbit antiserum against the Negroni agent, a strain of M. pulmonis, was markedly increased. It was reported by Purcell et al. (1966) that the metabolic inhibition titer of rabbit antisera against M. hominis, type I, M. arthritidis, M. salivarium and M. orale, type II was increased when unheated guinea pig serum was added to the medium. However, the addition of unheated guinea pig serum did not affect the metabolic inhibition titer of rabbit antiserum against M. orale, type I. Barker and Patt (1967) recently demonstrated the role of complement and antiserum in inactivation of M. gallisepticum. When unheated guinea pig serum was added to a culture of M. gallisepticum in broth containing M. gallisepticum antiserum produced in a rabbit the number of viable organisms fell rapidly from 10^6 to 10^2 organisms/0.2 ml. No change in the number of viable organisms occurred when heated guinea pig serum was added.

Since the metabolic inhibition titer of antiserum against various species of mycoplasma is either enhanced or unaffected by guinea pig serum, it was necessary to determine the effect of guinea

pig serum upon M. arthritidis, the organism studied in this thesis. The results of an experiment to determine the effect of guinea pig serum on the metabolic inhibition titer of antiserum against M. arthritidis are presented in Table 1.

Since there was no difference between the titer of antiserum in the presence of heated guinea pig serum or its absence, it seemed doubtful that the additional nutrient provided by the serum enhanced mycoplasma growth, which could lower the metabolic inhibition titer. However, the addition of unheated guinea pig serum increased in titer at least sixteen fold. This indicated a heat labile accessory factor was involved in the metabolic inhibition test when using M. arthritidis.

In addition to a heat labile factor, guinea pig serum also contained a heat stable factor which in low dilution inhibited M. arthritidis, strain 14152, from metabolizing arginine. This inhibition may be the result of several factors. First of all, the guinea pig serum, in very low dilutions may buffer the medium and prevent the pH from changing. Secondly, a nonspecific cidal agent or antibody in the guinea pig serum may kill the mycoplasma. A simple procedure was used to determine if the organisms in the metabolic inhibition test were viable after four days of incubation. No colonies appeared when a drop of the four-day culture containing guinea pig serum in a final dilution less than 1:42 was placed on PPLO agar and incubated at 37°C for four days. Colonies were detected when the same procedure was attempted with cultures containing heated or unheated guinea pig

TABLE 1

Effect of Guinea Pig Serum on the Metabolic Inhibition Titer of Rabbit Antiserum Against *M. arthritidis*, strain 14152

Test system*	Titer**
No guinea pig serum	400
Heated guinea pig serum (0.1 ml of 1:10)	400
Unheated guinea pig serum (0.1 ml of 1:10)	6400
0.25 ml of heated guinea pig serum; no rabbit antiserum	16
0.25 ml of unheated guinea pig serum; no rabbit antiserum	16

* Arginine broth medium

**Reciprocal of the highest dilution of serum which inhibited metabolism of arginine and color change of the indicator.

serum in a final dilution greater than 1:42. No viable organisms were detected in the tubes in which the indicator had not changed color. This indicated that more than "buffering" was involved in the inhibition of indicator color change.

Guinea pig serum was absorbed with M. arthritidis, strain 14152. The absorbed serum, diluted 1:16 caused inhibition of the metabolism of arginine by the mycoplasma, as did the guinea pig serum which had not been absorbed with M. arthritidis. This diminished the possibility that growth inhibiting antibodies for M. arthritidis, strain 14152, were present in guinea pig serum. It seemed likely a nonspecific inhibitor in the serum caused growth inhibition of the organisms.

In order to eliminate the possibility that the metabolic inhibition was caused by the guinea pig serum alone as opposed to M. arthritidis antiserum, fresh guinea pig serum was always used at a final dilution of 1:90 in all subsequent tests. At this dilution there was no metabolic inhibition by guinea pig serum alone.

Taylor-Robinson et al. (1966) presented evidence to indicate that when the horse serum constituent of the medium was heated for one hour at 56°C, the inhibition of M. pneumoniae by its homologous antiserum was significantly reduced. Since horse serum was a constituent of the metabolic inhibition test system, it was necessary to determine the effect of horse serum on the metabolic inhibition titer of antiserum prepared against M. arthritidis.

It can be seen in Table 2 that there was no difference in the metabolic inhibition titer of rabbit antiserum against M. arthritidis, strain 14152, when heated or unheated horse serum was used in the medium. This may be attributed to the age of the horse serum which was stored three to four weeks at 4°C. The labile factor which Taylor-Robinson detected in horse serum may be absent in the unheated horse serum due to loss during storage as well as in the heated horse serum. In any event, the addition of fresh guinea pig serum provided the heat labile accessory factor missing in horse serum.

TABLE 2

Effect of Heated Horse Serum on the Metabolic Inhibition of M. arthritidis, Strain 14152, by its Homologous Antiserum

Test system*	Titer**
Unheated horse serum	1600
Heated horse serum (1 hr. at 56°C)	1600

* Arginine broth without guinea pig serum

**Reciprocal of the highest dilution of serum which inhibited metabolism of arginine and color change of the indicator.

To further study the effect of heated horse serum and guinea pig serum on the metabolic inhibition titer of an antiserum against M. arthritidis, replicate titrations were performed with and without

the addition of fresh guinea pig serum and with heated or unheated horse serum. A comparison of the metabolic inhibition titers obtained is presented in Table 3.

In the results shown here unheated horse serum had no significant effect upon the metabolic inhibition titer while the addition of fresh guinea pig serum to the medium increased the metabolic inhibition titer four to eight fold. Unheated horse serum was used in subsequent tests since heated horse serum does not increase the metabolic inhibition titer of the antiserum. Unheated guinea pig serum, likewise, was used in metabolic inhibition tests but only at a final dilution of 1:90 since the serum, itself, causes inhibition of M. arthritidis when diluted less than 1:41.

II. ABSORBED RABBIT ANTISERUM

Rabbit antiserum was absorbed with M. arthritidis, strain 14152 antigen as described previously. A metabolic inhibition test was prepared using absorbed and unabsorbed antiserum prepared against M. arthritidis, strain 14152. The effect of unheated guinea pig serum on the metabolic inhibition titer of rabbit antiserum was studied. The results of the experiment are presented in Table 4.

The metabolic inhibition titer of unabsorbed antiserum was increased four fold by the addition of unheated guinea pig serum. The metabolic inhibition titer of rabbit antiserum absorbed with

TABLE 3

Effect of Heated Horse Serum and Fresh Guinea Pig Serum on the Metabolic Inhibition of M. arthritidis, Strain 14152, by its Homologous Antiserum

Test system*	Titer**
HHS*** with GPS****	12800
HS***** with GPS	6400
HHS without GPS	1600
HS without GPS	1600

*Arginine broth medium

**Reciprocal of the highest dilution of serum which inhibited metabolism of arginine and color change of the indicator

***Ten per cent heated horse serum (heated 1 hour at 56°C)

****Fresh guinea pig serum (final dilution of 1:90)

*****Ten per cent unheated horse serum

TABLE 4

Effect of Guinea Pig Serum on the Metabolic Inhibition of M. arthritidis, Strain 14152, by Absorbed and Unabsorbed Homologous Antiserum

Test system*	Titer**
Unabsorbed rabbit antiserum without guinea pig serum	1600
Unabsorbed rabbit antiserum with guinea pig serum	6400
Absorbed rabbit antiserum without guinea pig serum	50
Absorbed rabbit antiserum with guinea pig serum	100

* Arginine broth medium

**Reciprocal of the highest dilution of serum which inhibited metabolism of arginine and color change of the indicator

antigen prepared from M. arthritidis, strain 14152, was decreased 32 fold when fresh guinea pig serum was omitted from the test system. In the presence of guinea pig serum the reduction in metabolic inhibition titer after absorption was 64 fold. This indicated that antibody in the rabbit antiserum combined with its homologous antigen and was removed when the antiserum-antigen mixture was centrifuged. The addition of unheated guinea pig serum had little effect on the metabolic inhibition titer when most of the antibody had already been removed by absorption with its homologous antigen. However, when antiserum against M. arthritidis, strain 14152 was present (as in serum not absorbed) unheated guinea pig serum enhanced the titer. It is evident that both antibody and unheated guinea pig serum are necessary for maximum metabolic inhibition titers.

III. INDIRECT HEMAGGLUTINATION TEST

An indirect hemagglutination test was set up as described previously using M. arthritidis, strain 14152, and its homologous rabbit antiserum. This test was used to determine the amount of antigen required to sensitize tanned sheep erythrocytes and permit maximum hemagglutination of the cells by antiserum. The results of this test are presented in Table 5.

Complete agglutination of the erythrocytes occurred when the antigen was diluted as much as 1:64 and the rabbit antiserum diluted 1:12800.

TABLE 5

Indirect Hemagglutination Test Using M. arthritidis, Strain 14152, and its Homologous Rabbit Antiserum

Antigen dilution*	8	16	32	64	128	256	512	1024	2048	0
Serum dilution*										
50	+	+	+	+	+	∓	-	-	-	-
100	+	+	+	+	∓	-	-	-	-	-
200	+	+	+	+	+	∓	-	-	-	-
400	+	+	+	+	+	+	∓	-	-	-
800	+	+	+	+	+	+	∓	-	-	-
1600	+	+	+	+	+	+	+	∓	-	-
3200	+	+	+	+	+	+	+	∓	-	-
6400	+	+	+	+	+	+	∓	-	-	-
12800	+	+	+	+	∓	-	∓	-	-	-
25600	-	-	-	-	-	-	-	-	-	-
0	-	-	-	-	-	-	-	-	-	-

*Reciprocal of the dilution

+ = Complete agglutination of the sensitized sheep erythrocytes

- = No detectable agglutination of the sensitized sheep erythrocytes

∓ = 50% agglutination of the sensitized sheep erythrocytes

A large amount of sheep erythrocytes were tanned and sensitized with antigen, prepared from M. arthritidis, strain 14152, diluted 1:64. These cells were frozen as described previously and stored at -70°C until used. The frozen sensitized sheep erythrocytes were thawed and used in an indirect hemagglutination test. The results of this test are presented in Table 6.

Agglutination of the sensitized sheep erythrocytes occurred when the rabbit antiserum was diluted 1:6400. No detectable agglutination occurred when a rat antiserum was used.

An indirect hemagglutination test was set up using antigen prepared from M. arthritidis, strain 14152, and antiserum collected from a rat infected four weeks previously with M. arthritidis, strain 14152. The sensitized sheep erythrocytes (not frozen) did not agglutinate even at the lowest dilutions of antigen and homologous antiserum.

IV. MYCOPLASMA ISOLATES FROM RATS

Mycoplasma were isolated from tissues of autopsied Long-Evans, strain L white and black rats. Some of the mycoplasma isolates were tentatively identified by their physiological properties (Table 7); others by their colonial morphology (Table 8).

Organisms which had smooth, distinct central regions of growth into the agar were tentatively identified as M. arthritidis. Those with gold, rough, and indistinct centers were identified as M.

TABLE 6

Indirect Hemagglutination of Sensitized Sheep Erythrocytes* Using Rabbit and Rat Antiserum Against M. arthritidis, Strain 14152

Serum dilutions**	50	100	200	400	800	1600	3200	6400	12800	0
Test system										
Unsensitized SE*** with rabbit antiserum	-	-	-	-	-	-	-	-	-	-
Unsensitized SE with rat antiserum	-	-	-	-	-	-	-	-	-	-
Sensitized SE with rabbit antiserum	+	+	+	+	+	+	+	+	-	-
Sensitized SE with rat antiserum	-	-	-	-	-	-	-	-	-	-

*Sheep erythrocytes had been sensitized with antigen prepared from M. arthritidis, strain 14152 diluted 1:64 and frozen.

**Reciprocal of the dilution

***Sheep erythrocytes

TABLE 7

Physiological Properties of Mycoplasma Isolated From Rats

Isolate*	Colonial morphology	Arginine broth	Glucose broth	Egg yolk	Benzidine blood	TTC** aerob.	TTC** anaer.	Probable organism
29G	smooth, distinct center	+	-	-	-	-	+	M. arthritidis
29L	smooth, distinct center	+	-	-	-	-	+	M. arthritidis
31C	smooth, distinct center	+	-	-	-	-	+	M. arthritidis
31E	smooth, distinct center	+	-	-	-	-	+	M. arthritidis
31G	smooth, distinct center	+	-	-	-	-	+	M. arthritidis
31H	smooth, distinct center	+	-	-	-	-	+	M. arthritidis
31K	smooth, distinct center	+	-	-	-	-	+	M. arthritidis
31L	smooth, distinct center	+	-	-	-	-	+	M. arthritidis
32G	smooth, distinct center	+	-	-	-	-	+	M. arthritidis

TABLE 7 (Continued)

Isolate*	Colonial morphology	Arginine broth	Glucose broth	Egg yolk	Benzidine blood	TTC** aerob.	TTC** anaer.	Probable organism
32H	smooth, distinct center	+	-	-	-	-	+	M. arthritidis
33A	gold, indistinct center	-	+	+	+	+	+	M. pulmonis
33B	gold, indistinct center	-	+	+	+	+	+	M. pulmonis
33C	gold, indistinct center	-	+	+	+	+	+	M. pulmonis
33I	smooth, distinct center	+	-	-	-	-	+	M. arthritidis
33J	smooth, distinct center	+	-	-	-	-	+	M. arthritidis
33M	smooth, distinct center	+	-	-	-	-	+	M. arthritidis

*Number of rat and tissue from which the mycoplasma was isolated: saliva (A), nasal mucosa (B), lung (C), trachea (D), bladder (E), cecal material (F), liver (G), spleen (H), joint (I), lymph nodule (J), heart (K), kidney (L) or joint streaked onto sheep blood agar (M)

**Triphenyltetrazolium chloride agar incubated aerobically or anaerobically

TABLE 8

Colonial Characteristics of Mycoplasma Isolated From Rats Subject to Spontaneous Arthritis

Isolate*	Colonial morphology
29B	gold, rough, indistinct center
29C	gold, rough, indistinct center
29E	smooth, distinct center
29J	smooth, distinct center
29K	smooth, distinct center
30B	gold, rough, indistinct center
31B1	very small colony; no noticeable center
31B2	gold, rough, distinct center
31D	smooth, distinct center
31J	smooth, distinct center
32B1	very small colony; no noticeable center
32B2	gold, rough, indistinct center
32C	smooth, distinct center
33B2	smooth, distinct center (small colony)
33C2	smooth, distinct center (small colony)
33D1	smooth, distinct center
33D2	gold, rough, indistinct center
33E	smooth, distinct center
33G	smooth, distinct center
33H	smooth, distinct center

*Number of rat and tissue from which the mycoplasma was isolated; saliva (A), nasal mucosa (B), lung (C), trachea (D), bladder (E), cecal material (F), liver (G), spleen (H), joint (I), lymph nodule (J), heart (K), kidney (L).

pulmonis. Most of the rats harbored both M. arthritidis and M. pulmonis, the former being present in almost every tissue cultured. This indicated the possibility that septicemia occurred with M. arthritidis and distributed organisms in the various organs and tissues. M. pulmonis, however, was only isolated from saliva, nasal mucosa, and lung. This indicated that the organism was harbored in the oral and nasal cavities.

V. INTRAVENOUS INOCULATION OF RATS WITH M. ARTHRITIDIS

Sera collected from rats inoculated with M. arthritidis, strain 158, were tested for complement fixing antibody. The results of the complement fixation tests are presented in Tables 9-13. The degree of polyarthritis involvement was recorded in Tables 14-17.

The complement fixation titers of sera from animals injected intravenously with equal numbers of organisms were averaged and plotted against time in Figures 1-4. An average polyarthritis score is also shown in the same figures.

Complement fixing antibodies produced by rats in response to intravenous inoculation with M. arthritidis, strain 158, were correlated with the number of CFU of the organism injected. When $6x10^6$ or $6x10^7$ CFU of M. arthritidis were injected, arthritis was minimal and the complement fixation titers reached their maximum between fifty and sixty days after injection. However, when rats were inoculated with $6x10^8$ or $6x10^9$ CFU of M. arthritidis, complement fixation titers

TABLE 9

Complement Fixation Titers of Sera Obtained From Rats Injected Intravenously with $6x10^6$ CFU of M. arthritidis, Strain 158

Days after inoculation	Complement fixation titer*				
	54**	57	61	64	67
0	10	10	10	10	10
4	80	160	80	320	1280
8	320	160	40	1280	2560
14	160	2560	20	2560	5120
21		160	40	1280	2560
28	10	320	40	1280	320
35		5120	40	2560	640
42		2560	80	10240	640
49		10240	10	1280	640
56		20480	20	1280	2560
63		1280	10	160	640
92		320			320
147		320			320

*The reciprocal of the greatest dilution of serum which inhibited complete lysis of sheep erythrocytes.

**Animal number

TABLE 10

Complement Fixation Titers of Sera Obtained From Rats Injected Intravenously with $6x10^7$ CFU of M. arthritidis, Strain 158

Days after inoculation	Complement fixation titer*										
	47**	35	36	37	38	41	42	44	45	49	51
0	10	10	10	10	10	10	10	10	10	10	10
4	640	640	1280	1280	1280	2560	320	320	1280	5120	5120
8	640	5120	5120	640	640	5120	640		640	1280	2560
14		640	2560		2560			5120		2560	640
21	1280	5120	10240	2560	20480	640	1280	640	2560	1280	640
28	2560	2560	2560	1280	640	640	160	1280	160	1280	80
35	10240	1280	5120	20480	160	5120	640	5120	1280	5120	320
42	10240	5120	2560	20480	10240	2560	2560	20480	10240	1280	2560
49	2560	20480	1280	1280	10240	20480	1280	40960	10240	640	2560
56	5120	20480	40960	10240	5120	10240	2560		40960	1280	2560
63	5120	40960	2560	5120	1280	10240	160		10240		1280
92	5120					2560					

*Reciprocal of the greatest dilution of serum which inhibited complete lysis of sheep erythrocytes.

**Animal number

TABLE 11

Complement Fixation Titers of Sera Obtained From Rats Injected Intravenously with $6x10^8$ CFU of *M. arthritidis*, Strain 158

Days after inoculation	Complement fixation titer*				
	19**	23	24	28	33
0	10	10	10	10	10
4	2560	1280	2560	2560	1280
8	2560	5120	2560	5120	20480
14	20480	20480		20480	5120
21	10240	10240	2560	1280	2560
28	40960	1280	5120	5120	5120
35	20480	5120	2560	40960	1280
42	40960	2560	2560	10240	5120
49	40960	2560	2560	20480	10240
56	40960	10240	20480	40960	1280
63	5120	5120	5120	640	20480
92		640	320	2560	10240
147		160	320	640	2560

*Reciprocal of the greatest dilution of serum which inhibited complete lysis of sheep erythrocytes.

**Animal number

TABLE 12

Complement Fixation Titers of Sera Obtained From Rats Injected Intravenously with $6x10^9$ CFU of *M. arthritidis*, Strain 158

Days after inoculation	Complement fixation titer*				
	72**	73	74	78	81
0	10	10	10	10	10
4	80	10240			10240
8		1280	2560	2560	
14			1280	320	5120
21	1280	10240	1280	2560	20480
28	160	640	640	5120	1280
35	40960	20480	40960	20480	5120
42	20480	10240	10240	10240	20480
49	2560	20480	10240	1280	40960
56	20480	10240	40960	5120	20480
63	20480	10240	5120	2560	40960
92	2560		5120	640	
147	1280		2560	640	

*Reciprocal of the greatest dilution of serum which inhibited complete lysis of sheep erythrocytes.

**Animal number

TABLE 13

Complement Fixation Titers of Sera Obtained From Rats Injected Intravenously with 0.5 ml of PPLO Broth

Days after inoculation	Complement fixation titer*								
	3**	4	7	8	9	10	12	16	17
42	10	10	10	10	10	20	10	10	10
49	10	10	10	10	10	10	10	10	10
56	10	10	10	10	10	20	10	10	10

*Reciprocal of the greatest dilution of serum which inhibited complete lysis of sheep erythrocytes

**Animal number

TABLE 14

Polyarthritis Following Intravenous Injection of $6x10^6$ CFU of M. arthritidis, Strain 158

Days after inoculation	Polyarthritis score*				Average polyarthritis score***
	57**	61	64	67	
3	0	0	0	0	0
4	0	0	0	0	0
5	0	0	0	0	0
8	0	0	0	0	0
10	0	0	0	0	0
14	0	0	0	0	0
21	0	0	0	0	0
51	0	0	0	0	0

*The involvement of polyarthritis was determined by estimating the swelling of each joint and recording this on a scale of 1 to 4. Paralysis and partial paralysis were 1 and 0.5 respectively for each limb. The scores were totaled and recorded as polyarthritis.

**Animal number

***Obtained by averaging polyarthritis scores of individual animals at indicated times

TABLE 15

Polyarthritis Following Intravenous Injection of $6x10^7$ CFU of M. arthritidis, Strain 158

Days after inoculation	Polyarthritis score*											Average polyarthritis score***
	35**	36	37	38	41	42	44	45	47	49	51	
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	1.5	0	0	0	0	0	0	0	0	0	0
5	0	1	0	0	0	0	2	0	0	0	0	0.3
8	0	1	0.5	0.5	0	16	2	0	0	0	0	2
10	4	1	0.5	1.5	0	7	3	0	0	0	0	1.5
14	3	1	0.5	0.5	0	5	4	0	0	0	0	1.3
21	5	0.5	1	0.5	0	7	4	0	0	0	0	1.7
31	5.5	0	1.5	0	0	2.5	3	0	0	0	0	1.1
40	0	0	1	0	0	0.5	1	0	0	0	0	0.2
51	0	0	0	0	0	0	0	0	0	0	0	0

*The involvement of polyarthritis was determined by estimating the swelling of each joint and recording this on a scale of 1 to 4. Paralysis and partial paralysis were 1 and 0.5 point respectively for each limb. The scores were totaled and recorded as polyarthritis.

**Animal number

***Obtained by averaging polyarthritis scores of individual animals at indicated times

TABLE 16

Polyarthritis Following Intravenous Injection of $6x10^8$ CFU of M. arthritidis, Strain 158

Days after inoculation	Polyarthritis score* 23**	19	24	28	32	Average polyarthritis score***
3	4	0	1	0	0	1.0
4	2	2	1	2	5	2.4
5	3	2	0	2	5	2.4
8	5	2	0	2	3.5	2.5
10	6	2.5	3.5	2.5	4	3.2
14	2	2.5	2	2	1	2.0
21	3	2.5	0	0	4	2.0
31	0	1	0	0	1	0.4
40	0	0	1	2	0	0.6
51	0	0	0	0	0	0

*The involvement of polyarthritis was determined by estimating the swelling of each joint and recording this on a scale of 1 to 4. Paralysis and partial paralysis were 1 and 0.5 point respectively for each limb. The scores were totaled and recorded as polyarthritis.

**Animal number

***Obtained by averaging polyarthritis scores of individual animals at indicated times

TABLE 17

Polyarthritis Following Intravenous Injection of $6x10^9$ CFU of M. arthritidis, Strain 158

Days after inoculation	Polyarthritis score* 72**	73	74	78	81	Average polyarthritis score***
3	2	1	5	3.5	6	3.4
4	10	2	4	5	12	6.6
5	5.5	6	10	7	11	8.0
8	9	4	19	11	14	11.4
10	8	8	22	7	8	8.6
14	6	6	13	6.5	6	7.4
21	3	4.5	7	4	6	4.8
31	2	1.5	4	2	5	3.0
40	0	0	1	2	1	0.8
51	0	0	1	1	1	0.6

*The involvement of polyarthritis was determined by estimating the swelling of each joint and recording this on a scale of 1 to 4. Paralysis and partial paralysis were 1 and 0.5 point respectively for each limb. The scores were totaled and recorded as polyarthritis.

**Animal number

***Obtained by averaging polyarthritis scores of individual animals at indicated times

Figure 1. Average complement fixation titers of sera collected from rats injected with $6x10^6$ CFU of M. arthritidis, strain 158 and average polyarthritis score of the same animals

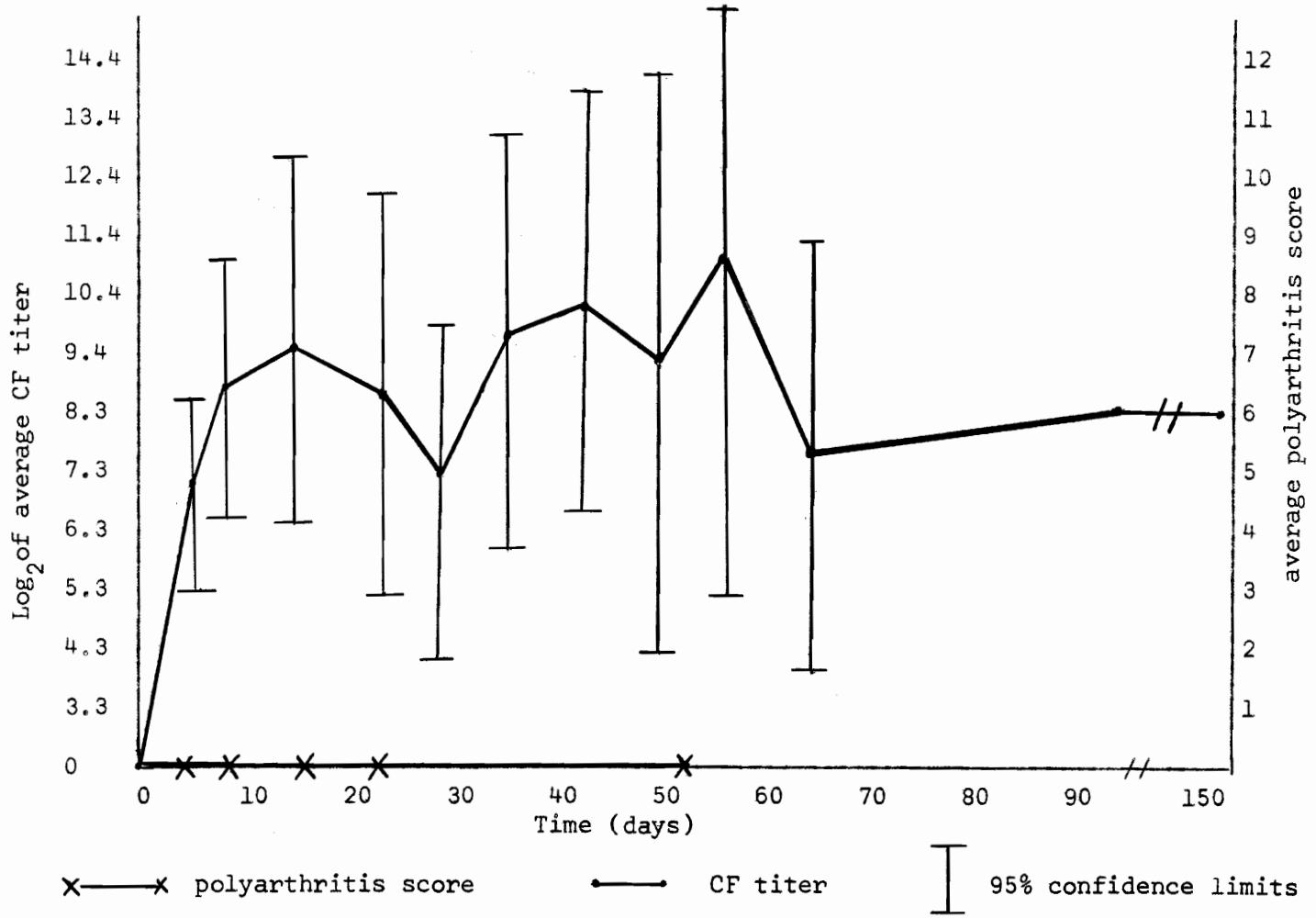


Figure 2. Average complement fixation titers of sera collected from rats injected with $1x10^7$ CFU of M. arthritidis, strain 158 and average polyarthritis score of the same animals

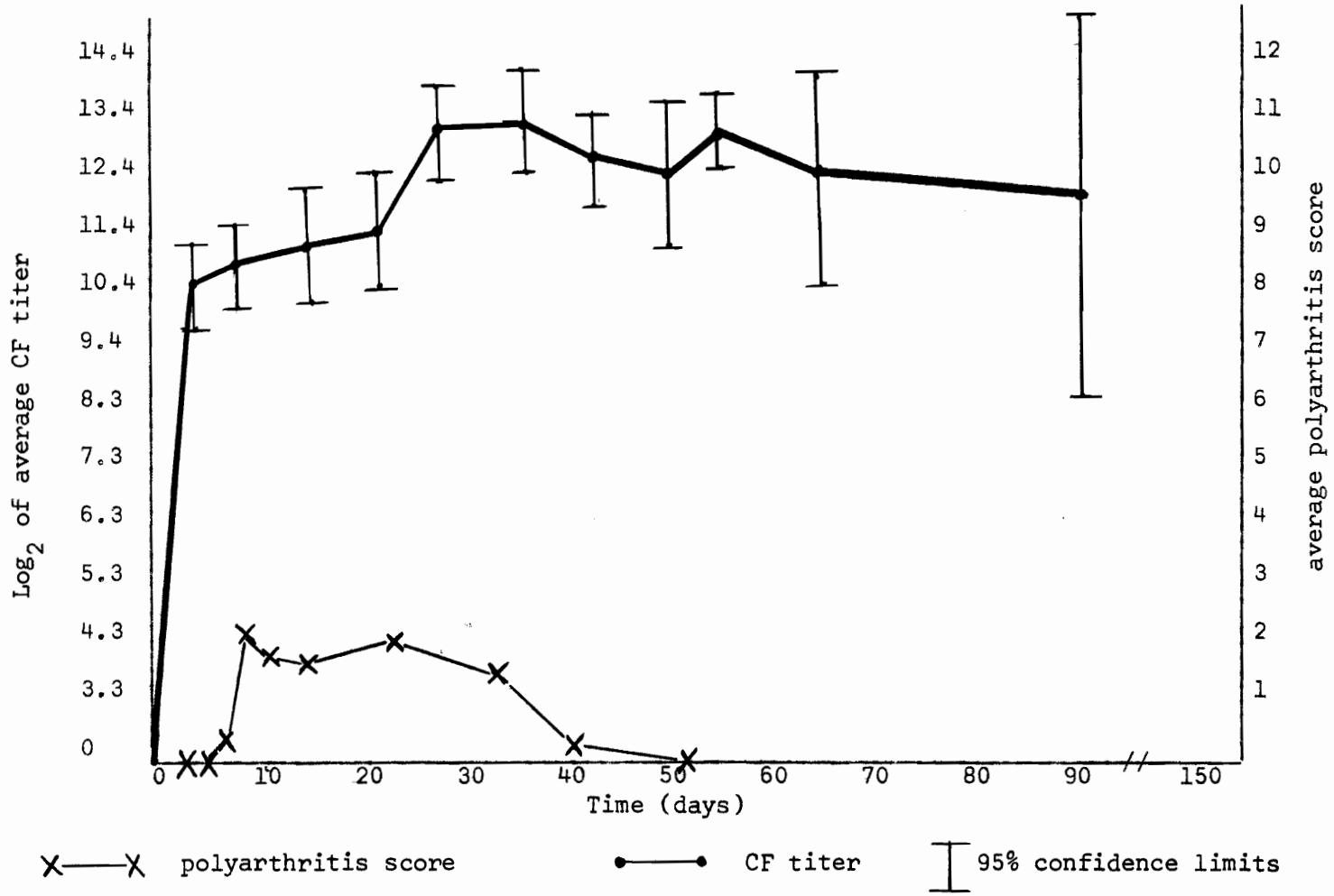


Figure 3. Average complement fixation titers of sera collected from rats injected with 6×10^8 CFU of M. arthritidis, strain 158 and average polyarthritis score of the same animals

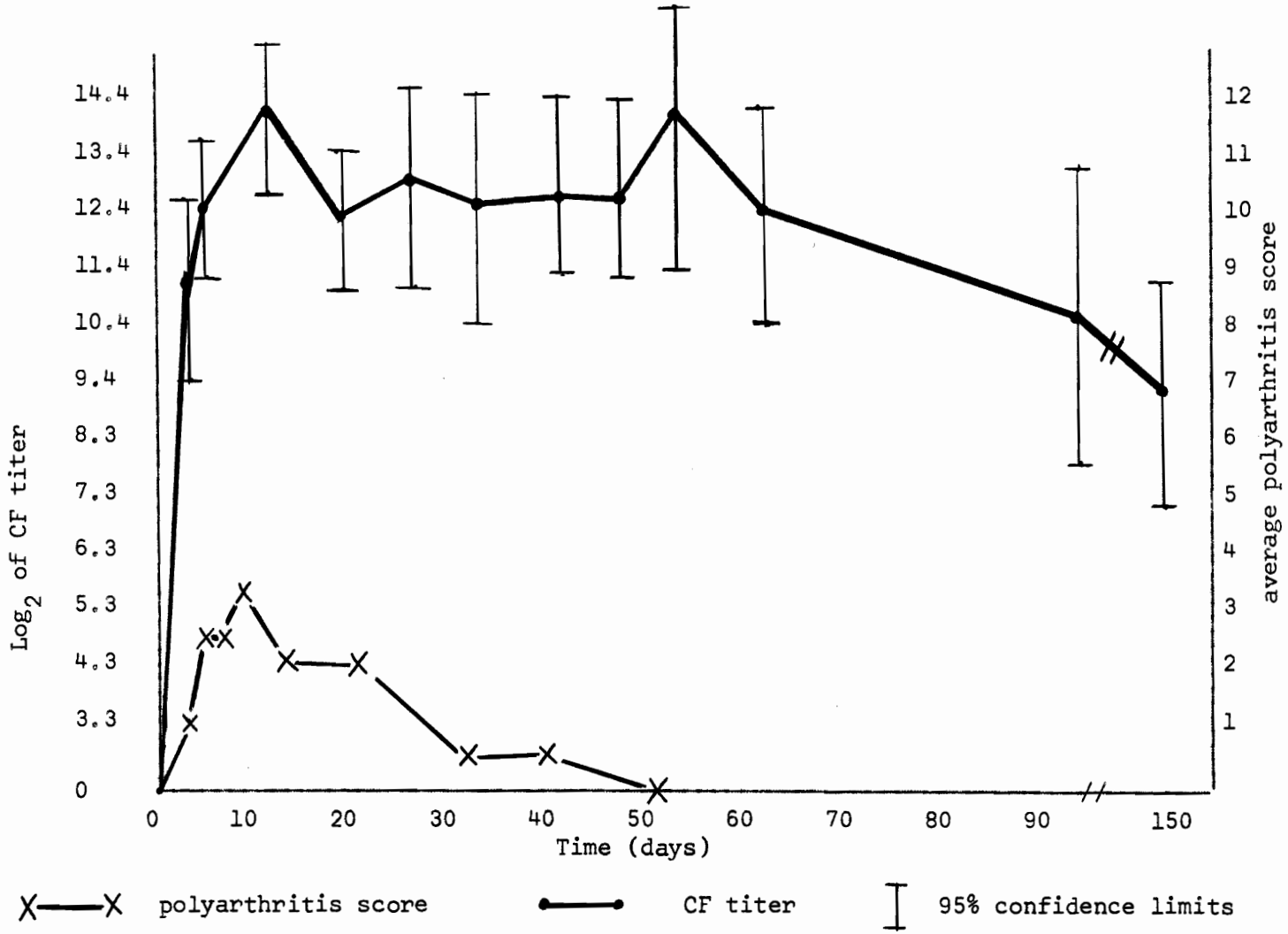
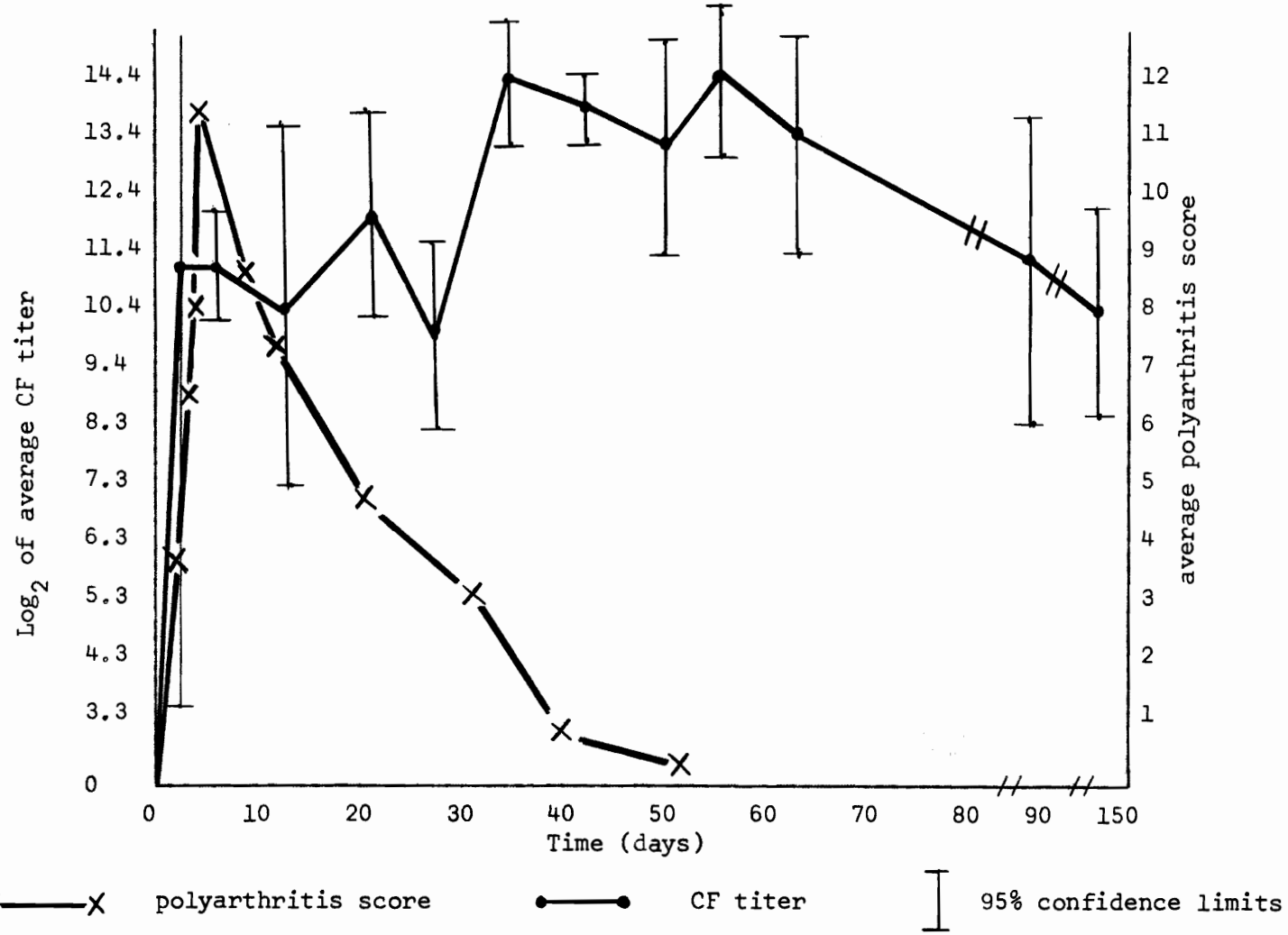


Figure 4. Average complement fixation titers of sera collected from rats injected with 6×10^9 CFU of M. arthritidis, strain 158 and average polyarthritis score of the same animals



rose to high levels within eight days after injection, at a time when polyarthritis was maximal; and these complement fixation titers remained at a high level through the sixtieth day.

Polyarthritis occurred on the third day after intravenous injection of the rats with the suspension of mycoplasma and reached a maximum between seven and fourteen days. It declined gradually and had completely disappeared by the sixth or seventh week. Here again, the response was directly related to the size of the inoculum. Animals injected with $6x10^9$ or $6x10^8$ CFU of the organisms had extensive polyarthritis which lasted five to six weeks; while polyarthritis in animals inoculated with $6x10^6$ or $6x10^7$ CFU was less severe and lasted three to six weeks. Individual animals within each group varied considerably. For example, some serum samples, collected 21 days after a rat was injected with $6x10^9$ CFU, fixed complement when diluted 1:1280; while serum samples from another rat injected with the same number of organisms fixed complement when diluted 1:10240. There was an eight-fold difference between the two titers.

A large number of rats was used in the experiment where rats were inoculated intravenously with varying numbers of M. arthritidis to offset biological variability. However, approximately 10% of the animals died when bled by cardiac puncture. This greatly reduced the number of animals being tested.

VI. CHALLENGE WITH M. ARTHRITIDIS

Nine weeks after the first injection of rats with M. arthritidis, the surviving animals were divided into two groups. Thirteen of the animals were used to complete the study on complement fixing antibody. Blood samples were collected approximately every two weeks and tested for complement fixing antibody. The remaining fourteen animals were challenged with 1.8×10^{10} CFU of M. arthritidis, strain 158. The animals, which had previously been injected with PPLO broth, were injected intravenously with either 1.8×10^{10} CFU of M. arthritidis, strain 158, or PPLO broth. Ten normal rats, weighing 350 to 400 grams (the weight of the animals previously inoculated with M. arthritidis), were also injected intravenously with 1.8×10^{10} CFU of M. arthritidis, strain 158. The severity of polyarthritis for each animal is shown in Tables 18-21.

One of fourteen animals which had been inoculated with M. arthritidis, strain 158, and challenged with the same organism nine weeks later contracted polyarthritis. This animal did not have polyarthritis after primary injection of M. arthritidis, but did have a low complement fixing antibody response which reached a maximum of 1:80 and fell to 1:10 prior to challenge with M. arthritidis. Several other rats had not exhibited polyarthritis on primary injection. They did not exhibit polyarthritis when challenged with M. arthritidis.

On primary injection of 6×10^7 and 6×10^8 CFU of M. arthritidis, strain 158, 80% and 100% of the animals, respectively, developed

TABLE 18

Polyarthritis Following Intravenous Injection of Normal Rats with 1.8×10^{10} CFU of M. arthritidis, Strain 158

Days after inoculation	Polyarthritis score*										Average polyarthritis score***
	86**	87	88	89	90	91	92	93	94	95	
3	22	21	23	25	21	22	31	12	14	28	19.8
4	44	38	33	51	42	46	63	20	25	53	41.5
8	54		49	75	48	77	79	41	48	81	61.3

*The involvement of polyarthritis was determined by estimating the swelling of each joint and recording this on a scale of 1 to 4. Paralysis and partial paralysis were 1 and 0.5 point respectively for each limb. The scores were totaled and recorded as polyarthritis.

**Animal number

***Obtained by averaging polyarthritis scores of individual animals at indicated times

TABLE 19

Polyarthritis of Rats Injected Intravenously with PPLO Broth and Challenged Nine Weeks Later with an Intravenous Injection of 1.8×10^{10} CFU of M. arthritidis, Strain 158

Days after challenge	Polyarthritis score*						Average polyarthritis score***
	4**	5	7	8	9	10	
3	37	42	24	28	21	8	26.7
4		80	67	40	55	22	53.0
8		99	82	25	54	30	58.0

*The involvement of polyarthritis was determined by estimating the swelling of each joint and recording this on a scale of 1 to 4. Paralysis and partial paralysis were 1 and 0.5 point respectively for each limb. The scores were totaled and recorded as polyarthritis.

**Animal number

***Obtained by averaging polyarthritis scores of individual animals at indicated times

TABLE 20

Polyarthritis of Animals Injected Intravenously with PPLO Broth and Challenged Nine Weeks Later with Another Intravenous Injection of PPLO Broth

Days after challenge	Polyarthritis score* 12**	16	17	Average polyarthritis score***
3	0	0	0	0
4	0	0		0
8	0	0		0

*The involvement of polyarthritis was determined by estimating the swelling of each joint and recording this on a scale of 1 to 4. Paralysis and partial paralysis were 1 and 0.5 point respectively for each limb. The scores were totaled and recorded as polyarthritis.

**Animal number

***Obtained by averaging polyarthritis scores of individual animals at indicated times

TABLE 21

Polyarthritis of Animals Injected Intravenously with M. arthritidis, Strain 158, and Challenged Nine Weeks Later with an Intravenous Injection with the Same Organism

Days after challenge	Polyarthritis score* 19**	21	35	36	37	38	42	45	49	61	73	81	83	Average polyarthritis score***
3	0	0	0	0	0	0	0	0	0	22	0	1	0	1.7
4	0	0	0	0	0	0	0	0	0	41	0	0	0	3
8	0	0	0	0	0	0	0	0	0	43	0	0	0	3

*The involvement of polyarthritis was determined by estimating the swelling of each joint and recording this on a scale of 1 to 4. Paralysis and partial paralysis were 1 and 0.5 point respectively for each limb. The scores were totaled and recorded as polyarthritis.

**Animal number

***Obtained by averaging polyarthritis scores of individual animals at indicated times

polyarthritis. Challenge of these animals with 1.8×10^{10} CFU (approximately 800 ED_{50}) did not induce polyarthritis.

Metabolic inhibition and complement fixation tests were set up with rat sera collected 28 days after inoculation of the rats with M. arthritidis, strain 158. The results of this test are presented in Table 22.

TABLE 22

Metabolic Inhibition and Complement Fixation Titers of Rat Sera Collected 28 Days After Inoculation of Rats with M. arthritidis

Animal number	MI titer*	CF titer**
19	10	40960
23, 36	10	1280
24, 28, 33	10	5120
34	10	2560
37, 38	10	640

*Reciprocal of the highest dilution of serum which inhibited color change of the indicator

**Reciprocal of the highest dilution of serum which inhibited complete lysis of sheep erythrocytes

It can be seen in Table 22 that rats inoculated with M. arthritidis did not produce detectable amounts of metabolic inhibiting antibody. Rat sera which had a high complement fixation titer had no metabolic inhibition titer.

DISCUSSION

Homologous antibody plus fresh guinea pig serum can cause lysis of gram-negative bacteria, spheroplasts, protoplasts and mammalian erythrocytes (Muschel and Jackson, 1966). Since cell membranes of mycoplasma are morphologically similar to membranes of mammalian cells (Domermuth et al., 1964), complement may play an important role in the inactivation of mycoplasma.

From the results presented in this thesis it can be seen that the metabolic inhibition titer of rabbit antiserum prepared against M. arthritidis, strain 14152, was greatly increased by the addition of fresh guinea pig serum. When fresh guinea pig serum heated at 56°C for 30 minutes was used, the metabolic inhibition titer was no higher than when guinea pig serum was omitted from the metabolic inhibition test system. This confirmed the observation of others that a heat labile accessory factor in fresh guinea pig serum increased the metabolic inhibition titer of antiserum prepared against various mycoplasma species (Barker and Patt, 1967; Purcell et al., 1966; Taylor-Robinson et al., 1966).

It was found that metabolic inhibition tests in which the indicator had not changed color did not contain viable organisms. In other words, the mycoplasma which were inoculated into the metabolic inhibition test system were not only inhibited in the

metabolism of arginine, as indicated by failure of the indicator to change color, but were apparently killed. This adds support to the work of Barker and Patt (1967) who reported that M. gallisepticum was killed by the addition of homologous antiserum and complement.

The addition of fresh guinea pig serum to antibody-sensitized gram-negative bacteria results in the release of ribonucleic acid and deoxyribonucleic acid, presumably by disrupting permeability control mechanisms of the cell (Spitznagel, 1964). A heat labile substance in fresh guinea pig serum causes lesions in the cell membranes of sheep erythrocytes which have been sensitized with antiserum (Borsos et al., 1964). It is possible that a similar mechanism is involved in the metabolic inhibition test. Antibody could attach to the mycoplasma, then in the presence of fresh guinea pig serum, result in increased permeability of the cell or lysis of the cell membrane. The effects described could result in loss of internal components and inability of affected organisms to carry on normal metabolic processes.

Both heated and fresh guinea pig serum in low dilution were shown to prevent metabolism of arginine by M. arthritidis. It seemed possible that antibodies in low titer in the fresh guinea pig serum reacted with M. arthritidis causing inhibition of metabolism of arginine by the organism. The metabolism of arginine by M. arthritidis, however, was also inhibited when fresh guinea pig serum in low

dilutions was absorbed with antigen prepared from M. arthritidis, strain 14152. This diminished the possibility that metabolic inhibiting antibodies for M. arthritidis were present in fresh guinea pig serum. It seemed likely a nonspecific inhibitor in the serum caused metabolic inhibition of the organisms.

The metabolic inhibition titer of unabsorbed rabbit antiserum against M. arthritidis, strain 14152, was increased four fold by the addition of fresh guinea pig serum. Rabbit antiserum which had been absorbed with M. arthritidis, strain 14152, antigen no longer inhibited metabolism of arginine to the extent of unabsorbed antiserum. The titer decreased 64 fold even when fresh guinea pig serum was added to the test system. This indicated that specific antibody for M. arthritidis was removed when the antigen-antiserum mixture was centrifuged. The addition of fresh guinea pig serum had little effect on the metabolic inhibition titer when most of the antibody had already been removed by absorption with its homologous antigen. However, when unabsorbed antiserum against M. arthritidis, strain 14152, was present, fresh guinea pig serum enhanced the titer. It is evident that both antibody and fresh guinea pig serum are necessary for maximum metabolic inhibition titers.

It is postulated that intravenous injection of M. arthritidis into the rat results in polyarthritis when the organisms survive natural defenses of the host and become lodged in the joint tissue. There they grow and possibly produce a substance which accumulates

and causes inflammation. This substance may be enzymes or toxins produced by the mycoplasma or the mycoplasma, themselves. Since rats subject to spontaneous polyarthritis harbor M. arthritidis and exhibit signs of polyarthritis only when subjected to unfavorable environmental conditions or injury, it seems likely that stress permits or even enhances the growth of M. arthritidis. In the experimental situation where large numbers of mycoplasma are required to produce polyarthritis, a hypothetical substance postulated as responsible for arthritis could accumulate at a rate dependent upon the number of organisms injected. Polyarthritis would then occur in a shorter period of time and be more severe when large numbers of mycoplasma are injected into the rat. After intravenous injection of M. arthritidis the rat may produce antibodies against the organisms or their products. Polyarthritis became most severe 7 to 10 days after the rats were inoculated. Antibody may eventually aid in the animals' recovery and subsequent resistance to challenge with M. arthritidis, since preliminary experiments disclosed that uninoculated rats could be passively protected against an intravenous challenge inoculum of live M. arthritidis when given serum from rats that had recovered from arthritis. In spite of the demonstration of passive protective properties in such rat serum, no metabolic inhibiting antibodies could be demonstrated in the serum. Thus an enigmatic situation may exist wherein animals are passively protected by serum from rats that recovered from arthritis; yet, no antibody could be

demonstrated that correlated with the protective properties of the serum. The serum used in the passive protection tests had high titers of complement fixing antibody but the latter may not necessarily be related to passive protection of the rat from arthritis. It is possible that the passive protective properties of the rat serum may have been due to an antibody not detectable by the metabolic inhibition test. It also seems reasonable to speculate that the complement fixing antibodies present in the serum have nothing to do with the ability of these organisms to metabolize arginine. The site of inhibition of metabolism of arginine by metabolic inhibiting antibody presumably is the mycoplasma cell membrane (Purcell, 1966). Recent studies of human diploid fibroblasts in tissue culture suggest that contact of one fibroblast with another is sufficient to inhibit cell metabolism. The cell membrane plays an important role in regulating the synthesis of cell constituents and factors which influence the cell membrane also affect cell metabolism (Eagle, 1965).

The complement fixation titer of sera collected from rats injected intravenously with M. arthritidis, strain 158, was directly related to the number of organisms injected. The highest complement fixation titer occurred in rats which were inoculated with $6x10^9$ CFU of M. arthritidis. Relatively low titers occurred in rats inoculated with $6x10^6$ CFU of M. arthritidis. Approximately $6x10^7$ CFU of M. arthritidis injected intravenously were necessary to cause polyarthritis

in rats. The severity of polyarthritis is directly proportional to the number of organisms injected into the rat. The most severe polyarthritis occurred in rats inoculated with $6x10^9$ CFU.

Since large inocula of M. arthritidis are required for polyarthritis to develop and since polyarthritis does not occur until three to four days after injection of the organisms, it seems plausable that the organisms produce a substance which must accumulate to cause polyarthritis. This substance may be part of the mycoplasma or an exotoxin produced by them. Mycoplasma have recently been shown to produce toxins. Thomas (1967) reported that M. neurolyticum, the agent responsible for rolling disease in mice and rats, produces a filterable exotoxin in broth cultures. M. gallisepticum (Thomas, 1967) which has caused outbreaks of encephalitis in turkeys may contain or produce a neurotoxin which when injected intravenously into turkeys has a toxic action on the arteries of the brain.

Spontaneous polyarthritis in rats occurs only when the animal has been subjected to environmental stress. It is possible that mycoplasma organisms harbored in the tissue of the rat grow in necrotic tissue producing the substance which causes polyarthritis. Experimental rats, which had been injected intravenously with M. arthritidis, were resistant to intravenous challenge with the same organism. Resistance may be a result of antibody formed in response to products of the mycoplasma. It was shown that complement fixing antibodies were present within four days after intravenous injection

of rats with M. arthritidis. These antibodies did not protect the animals from polyarthritis since severe arthritis occurred when the complement fixation titer was rising. When $6x10^6$ or $6x10^7$ CFU of M. arthritidis were injected, arthritis was minimal and the complement fixation titers reached their maximum between 50 and 60 days after injection. However, when rats were inoculated with $6x10^8$ or $6x10^9$ CFU of M. arthritidis, complement fixation titers rose to high levels within eight days after injection at a time when polyarthritis was maximal and these complement fixation titers remained at a high level through the sixtieth day. Antibody against the substance or substances which caused polyarthritis may not be measured by the complement fixation test since a complement fixation titer occurred even before arthritis developed. According to Raffel (1961) complement fixing antibody has little or no relationship to the mechanisms of acquired resistance in bacterial infections. The observations reported here certainly support Raffel's contention. Animals are known to respond to some antigenic stimuli by the production of antibody which cannot be revealed by the serologic tests employed for the purpose (Raffel, 1961). Whether or not such antibody could function in a protective capacity in vivo without serological activity in vitro is not known. It should be pointed out, however, that the production of the antiserum against M. arthritidis, strain 14152, in the rabbit made use of multiple injections of killed organisms given with Freund's complete adjuvant. This rabbit antiserum did exhibit metabolic inhibiting antibodies in

high titer. The rat antiserum was produced following a single intravenous injection of living mycoplasma. Thus, the observations discussed here could have been due to differences in route of injection, differences in type of antigen and differences in the species of animal used.

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